(54) Title: NATIVE-STATE METHOD AND SYSTEM FOR DETERMINING VIABILITY AND PROLIFERATIVE CAPACITY OF TISSUES *IN VITRO*

(57) Abstract

The present invention relates to a method of using an in vitro culture system to measure the cellular proliferative capacity and cellular viability of human tissues, particularly tumor tissues. The invention also describes the use of the method to evaluate the effectiveness of an antineoplastic drug in inhibiting tumor cell proliferation or viability.

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NATIVE-STATE METHOD AND SYSTEM FOR DETERMINING VIABILITY AND PROLIFERATIVE CAPACITY OF TISSUES IN VITRO

DESCRIPTION

Technical Field

The present invention relates to a method of using an in vitro culture system to measure the cell proliferation and cell viability of human tissues, particularly tumor tissues, and to measure the efficacy of anti-neoplastic drugs upon the proliferation and viability of the cultured cells.

Background

Cancer is a disease involving inappropriate cell division. A realistic model is greatly needed to understand the biology of altered proliferation in cancer as compared to normal tissue and to use information on proliferation capacity as a basis of cancer prognosis and treatment.

Measurements of proliferation capacity of tumors currently are obtained by thymidine-labeling index (TLI), by flow cytometric measurements of cells presumed to be in S phase, or by measuring a nuclear antigen, Ki-67, found in at least certain proliferating cell types. Meyer et al., Breast Cancer Rest. Treat., 4:79-88 (1984); McDivitt et al., Cancer, 57:269-76 (1986); and McGurrin et al., Cancer, 59:1744-50 (1987). Whichever method is used, the results obtained show that the higher the S-phase fraction is, the poorer the prognosis. Clinical studies utilizing the TLI procedure have been successful in identifying and determining therapy of a subgroup of lymphnode-negative women with breast cancer having a 48% relapse rate.

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Meyer et al., Cancer, 51:1879-86 (1983). There is
therefore great potential value for cancer
prognosis, therapy, and biology in determining the
proliferative capacity of tumors.

5   However, as important as the measurement
of the TLI seems to be, current methods of
measuring the TLI are impractical and are not
physiological. For breast tumors, assays must be
conducted within approximately 2 hours of surgery,

10   precluding a central laboratory from carrying out
the measurement. Generally, the TLI is measured
under very high atmospheric pressure in a salt
solution to allow penetration of ^{3}H-thymidine into
the tissue. Under these conditions the tumor loses

15   viability after a few hours and in many cases it
must be assumed that cells capable of cycling are
not measured since the time of measurement is so
much less than the generation time of the
asynchronous cells within the tumor. With regard

20   to other human tumor types, there is very little
information regarding measurement of proliferation
capacity of surgical specimens.

       While flow cytometry provides a more
rapid method of measuring cell cycle kinetics and

25   cells can also be assessed for aneuploidy, it
presents the following technical problems: (i)
Dissociation, either mechanically or enzymatically,
into a single-cell suspension is required,
resulting in loss of ability to observe tissue

30   architecture and the potential selective loss of
one or more specific populations of cells. Full
evaluation of all the heterogeneous cell types of
an individual tumor, including their spatial
organization, is of obvious importance in the

35   development of prognostic tests. (ii) Flow
cytometry does not unambiguously distinguish

between S-phase diploid cells and aneuploid resting or nonviable cells. This becomes an important issue, as studies have demonstrated that tumor cell subpopulations that are enriched in aneuploid cells are largely nonviable by dye-exclusion analysis. Frankfurt et al., Cytometry, 5:71-80 (1984); Slocum et al., Cancer Res., 41:1428-34 (1981); and Ljung et al., Proc. Am. Assoc. Cancer Res., 28:34 (1987). (iii) In addition, the S-phase factions of diploid tumors are likely to be underestimated by flow cytometry due to contamination with non-proliferating, nonneoplastic cells. The invasive capacity of diploid cells in vitro from primary breast carcinomas has been clearly demonstrated. Smith et al., Proc. Natl. Acad. Sci. USA, 82:1805-9 (1985).

The nuclear antigen Ki-67 seems to be present in proliferating breast cancer cells [McGurrin et al., Cancer, 59:1744-50 (1987)], but its relevance to other tissue types is not yet known.

Perhaps most importantly, these techniques measure cells in S phase at a single point in time (flow cytometry, Ki-67) or after a very short labeling time (TLI). Thus, these measurements preclude an estimation of the total cell growth fraction of the tumor which may well reflect a more accurate measurement of the proliferative capacity of the tumor.

Importantly, none of the above methods have been applied to systematically measure the proliferation capacity of normal tissues, in particular in comparison with adjacent tumor tissues, nor have the above methods been applied systematically in comparative measurements of tumor proliferation in the presence of anti-proliferative

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therapeutics.

Brief Summary of the Invention

The present invention provides in
improved in vitro assay system to determine
cellular proliferation and/or cellular viability of
cells from patient biopsies. It has been found
that the determinations as to cellular
proliferation and viability made according to the
present invention accurately predict the grade,
stage and overall aggressiveness of a tumor.

A method for measuring the tumor specific
effects of an agent on cell proliferation and/or
viability is also contemplated. The method
comprises histoculturing, in separate containers,
first and second portions each of tumor tissue and
normal tissue samples. The normal tissue sample is
from a tissue analogous to the stem cells of the
tumor. The histocultured samples are exposed to
one or more concentration of an agent whose effects
are being examined. The exposed samples are then
treated (contacted) with either a proliferation
marker (DNA synthesis marker) and cultured
(histocultured) for a predetermined period of time.
The percent of cell having marker incorporated
therein is then determined, and, by comparing the
results obtained from the tumor cells with the
analogous normal cells, the tumor specific effect
of the agent on cell viability and/or proliferation
is determined.

Detailed Description of the Invention

The present invention provides a method
for measuring the effect of an agent on the
viability and/or proliferation of cells in their
native environment, i.e., as part of a tissue. In
carrying out such "drug" sensitivity measurements,
the effect on the cells of the tissue to exposure

to one or more given dose levels of one or more given drugs is measured by individually culturing aliquots of the same explanted tissue using a plurality of containers. One of the aliquots serves as a control and is quantitatively assayed for cell viability and/or proliferation in the absence of drug exposure. Each of the other aliquots is exposed to the drug at a different dose level and then quantitatively assayed for viability and/or proliferation using a protein-synthesis marker or DNA-synthesis marker, respectively. That is, the number of viable cells in each tissue sample is indicated by the number of cells having metabolically incorporated the protein-synthesis marker into the cellular protein. Similarly, the number of proliferating cells is indicated by the number of cells having metabolically incorporated the DNA-synthesis marker into the cellular DNA.

Metabolic markers for cellular protein synthesis are well known in the art and include radioactively labeled amino acids such as ^{35}S-methionine, ^{14}C-alanine, ^{14}C-glycine, ^{14}C-glutamic acid, ^{14}C-proline, ^{3}H-leucine, ^{3}H-serine and the like.

Metabolic marker for cellular DNA-synthesis are also well known in the art and include radioactively labeled nucleotides such as ^{3}H-deoxythymidine, ^{3}H-deoxyadenine, ^{3}H-deoxyguanine, ^{3}H-deoxycytosine and the like.

The various types of tissues to which the present invention is applicable include normal tissues as well as primary or metastatic tumors, including solid tumors (both carcinomas and sarcomas) and the like.

The various types of carcinomas (adeno, squamous and undifferentiated variants for

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carcinomas of various sites), to which the present
assay are applicable include, for example, adrenal,
bladder, breast, colon, kidney, lung, ovary,
pancreas, prostate, thyroid, upper airways (head
and neck), uterus (corpus and cervix), bile ducts,
choriocarcinoma, esophagus, liver, parathyroid,
rectum, salivary glands, small bowel, stomach,
testis, tongue and urethra. The various types of
sarcomas and other neoplasms to which the present
assay are applicable include, for example, diffuse
lymphomas, Ewing's tumor, Hodgkin's disease,
melanoma (melanotic and amelanotic), multiple
myeloma, nephroblastoma (Wilm's tumor),
neuroblastomas, nodular lymphomas,
rhabdomyosarcoma, angiosarcoma, brain tumors
(gliomas), chondrosarcoma, dysgerminoma,
fibrosarcoma, leiomyosarcoma, liposarcoma,
medulloblastoma, mesothelioma, osteosarcoma,
retinoblastoma and thymoma.

Typically, the tissue whose cell
viability and/or proliferation characteristics are
to be determined is explanted by an a septic
surgical procedure and a portion thereof is divided
into sections having a volume of about 0.5 to about
10, preferably a volume of about 1.0 to about 8.0,
more preferably 1.0 to 2.0 cubic millimeters.

When tumors are being assayed, it is
important to examine multiple portions of the tumor
in separate assays because tumors are very
heterogeneous.

After cubing, the explanted tissue is
divided into aliquots, typically at least about
six, one of which is typically designated a control
that receives no exposure or contact with the
compound being examined. The aliquots are then
histocultured on a hydrated extracellular-matrix-

containing gel so that the three-dimensional integrity of the tissue is maintained.

Tissue culture grade extracellular-matrix- containing gels suitable for histoculture are well known in the art and include those described by U.S. Patent No. 4,060,081 to Yannas et al., that available under the tradename of Gelfoam from Upjohn, and the like.

Various liquid tissue culture nutrient media capable of supporting tissue cell growth are known in the art. The medium used can either be serum-containing or serum-free with additives such as insulin, transferrin, selenium, estradiol and the like. A culture medium found to be particularly suitable in the present invention in Eagle's minimal essential medium (MEM) [Eagle, Science, 122:501 (1955) and Eagle, Science, 130:432 (1959)].

The tissues are typically histocultured for at least 2-5 days preferably about 3-4 days prior to exposing the cells therein to the agent being examined. Culturing is typically performed in a humidified atmosphere at a temperature corresponding to that of the body temperature of the animal from which the tissue sample came, e.g. 37°C for human tissue samples.

Drug exposure of the cells for the purposes of the viability and/or proliferation measurements is preferably carried out prior to treating the tissue samples with the protein-synthesis and/or DNA-synthesis markers. The procedure involves incubating the tissue sample with a predetermined amount (determinate concentration) of the drug for a predetermined period of time (determinate time period) and thereafter separating the tissue sample from the

drug, and preferably washing the tissue sample free
of residual drug.

The phrase "drug exposure dose level", as
used herein, refers to the quantitative product of
the drug concentration (e.g. in μl) and the time of
the exposure period (e.g., in hours or minutes).
The drug concentrations and exposure times are
typically calculated from pharmacological data to
the simulate <u>in vitro</u> the drug exposure dose level
achieved <u>in vivo</u>. Typically, it has been found
that the drug exposure dose level required in
carrying out the drug sensitivity measurements in
accordance with the assay of the present invention,
is at a maximum of only 5 to 10% of the clinically
achievable drug exposure dose level for the known
anticancer drugs which have been tested in the
present system.

The drug sensitivity measurements as
described above can be carried out in a manner
which enables the determination, for any given
drug, of a "drug sensitivity index", which is
indicative of the antineoplastic activity of the
given drug against the specific human tumor from
which the explanted cells were obtained. This
procedure involves carrying out the drug
sensitivity measurements of a plurality of dose
levels extending over a multi-log range, and then
using the results of these measurements to plot a
curve of percent survival (the percentage of the
assay count resulting from drug exposure versus the
assay count of the control in the absence of drug
exposure) versus drug exposure dose level. The
"drug sensitivity index" of the given drug is then
quantitated by measuring the area under such curve
out to a defined upper limit which is correlated to
the clinically achievable peak drug exposure dose

level for that drug.

The sensitivity index obtained in the above-described manner is highly indicative of the antineoplastic activity of the drug against the specific human tumor from which the explanted cells were obtained, with a low sensitivity index indicating high antineoplastic activity.

After histoculturing the cells in the presence of the agent being examined (about 1 to 4 days) the samples are treated (cultured in the presence of) the proliferation and/or viability marker.

After the cells of the tissue samples have been exposed to the drug(s), treated with the proliferation and viability markers and subsequently histocultured, the samples are typically fixed in the tissue fixative such as formalin, embedding in paraffin or the like and sectioned on a microtine. When the viability and/or proliferation markers contain radioactive labels, the sections are then treated with nuclear-track emulsion such as NTB (Kodak), and developed.

The sections are then assayed for the percentage of cells positive for the presence of the viability of proliferation marker. When radioactive labels and nuclear-track emulsions are used, this can be accomplished by light-polarized microscopy. In preferred embodiments, the magnified image can be digitized by processing it through a video camera operatively linked to a computer capable of digitizing the image for analysis.

Preliminary evidence indicates that the in vitro assay system of the present invention has utility for the in vitro prediction of clinical response to cancer chemotherapy, as well as the

screening of new anticancer drugs for clinical
trial. For example, in treating a specific patient
for a specific tumor, the explanted cells obtained
from a biopsy of such specific tumor can be assayed

5   in accordance with the present technique, a drug
sensitivity measurements can be carried out for a
plurality of different anticancer drugs which are
potentially clinically effective for the
chemotherapeutic treatment of the specific tumor.

10   After determining the relative drug sensitivity
indices for each of the various drugs tested, these
sensitivity indices may be used for predictably
selecting the most promising effective of the drugs
to be used for the chemotherapeutic treatment. In

15   preliminary clinical trials of this technique, both
retrospective and prospective, the correlation
found between the in vitro prediction and the in
vivo response was impressively high, approaching
100%.

20   In another embodiment, the present
invention contemplates a method of determining the
"growth factor index" of a tumor. The growth
factor index of a tumor correlates to the tumor's
in vivo grade and stage, i.e., the in vivo

25   aggressiveness of the tumor, particularly in breast
and ovarian carcinomas.

           The growth factor index is determined by
histoculturing, as previously described, a sample
of tumor cells. The cells are then treated with a

30   proliferation marker such as one of the before-
described DNA-synthesis marker, e.g., ^{3}H-
deoxythymidine. The treated cells are cultured for
a predetermined period of time and then the percent
of sample cells containing the proliferation marker

35   incorporated into the cellular DNA is determined,
that percentage representing the tumor's intrinsic

growth factor index.

In another embodiment, the present invention contemplates a kit for the in vitro determination of viability and/or proliferation of cells as described herein. The kit contains, in an amount sufficient for at least one assay, an agent to be tested for its effects on viability and/or proliferation, a viability and/or proliferation marker, and a container in which to perform the assay.

Examples

The following examples are given for illustrative purposes only and are not intended to be limiting unless otherwise specified.

1. Cell Proliferation

Various normal and tumor tissue specimens were ex-planted from human patients as described by Freeman et al., Proc. Natl. Acad. Sci. USA, 83:2694-98 (1986); and by Vescio et al., Proc. Natl. Acad. Sci USA, 84:5029-33 (1987). (The teachings of all of the references cited herein are hereby incorporated by reference). Briefly, after tissues were surgically removed, they were divided into 1- to 2-mm-diameter pieces and placed on top of previously hydrated extracellular-matrix-containing flexible gels derived from pigskin (Gelfoam, Upjohn) to form a three-dimensional culture. Eagle's minimal essential medium (MEM) containing Earle's salts, glutamine, 10% fetal calf serum, nonessential amino acids, and the antibiotics garamycin and claforan was added to the cultures such that the upper part of the gel was not covered, and cultures were maintained at 37 degrees C in a carbon dioxide incubator to allow the explanted tissue specimens to grow.

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Cells within the three-dimensional
cultures capable of proliferation were labeled by
administration of a combination of ^{3}H-thymidine and
^{3}H-deoxyuridine (2 μCi each; 1 Ci=37GBq) (Vescio et
5   al., Proc. Natl. Acad. Sci. USA, 84:5029-33, 1987)
for 4 days after 10-12 days in culture. Cellular
DNA is labeled in any cells undergoing replication
within the tissues. After 4 days of labeling, the
cultures were washed with phosphate-buffered
10   saline, placed in histology capsules, and fixed in
10% Formalin. The cultures were then dehydrated,
embedded in paraffin, and sectioned by standard
methods, and the sections were placed on slides.
            The sections on the slides were then
15   deparaffinized and prepared for autoradiography by
coating with Kodak NTB-2 emulsion in the dark and
exposed for 5 days, after which they were
developed. After developing and rinsing, the
sections were stained with hematoxylin and eosin.
20   The sections were then analyzed by
determining the percentage of cells undergoing DNA
synthesis in the various tissue cultures, using a
Nikon or Olympus photomicroscope fitted with an
epi-illumination polarization lighting system.
25   Replicating cells were identified by the presence
of silver grains, visualized as bright green in the
epi-illumination polarization system, over their
nuclei due to exposure of the NTB-2 emulsion to
radioactive DNA. The above procedures produce a
30   histological autoradiogram showing cellular
proliferation of the cells in a tumor explant
tissue.
            The large majority of tumors cultured in
the native-state system demonstrate at least some
35   areas of high cellular proliferation and are
intratumorally heterogeneous with regard to

proliferation capability. Tumors tested include
tumors of the colon, ovaries, pancreas, bladder,
kidney, brain, and parotid, and also include small-
cell lung carcinoma and Ewing sarcoma. In all
cases, three-dimensional tissue organization
representative of the original tissue is maintained
throughout the culture period. A high degree of
detection of radiolabeled proliferating cells is
afforded by the epi-illuminescence polarization
microscopy, which enhances detection of the
autoradiographic exposed silver grains by the
scatter of incident polarized light.

The proliferation capacity of a
metastatic colorectal tumor exhibited high labeling
in culture. More than 90% of the cells in the
observed culture preparation had proliferated
during the labeling period of this relatively
undifferentiated colon metastasis to the liver.

The proliferation capacity of a small-
cell lung tumor exhibited the maintenance of the
two major classes of oat cell types: the classic
small cells and the more elongate fusiform cell
types, each having a high degree of cell
proliferation.

The proliferation capacity in ovarian
carcinoma consistently exhibited an extremely high
index of proliferation of the epithelial cells
while the stromal cells remained quiescent. The
histological autoradiogram showed the high
proliferative capacity of the ovarian carcinoma
cells which have invaded the supporting gel matrix.
This invasive behavior may mimic the way ovarian
tumors frequently invade the peritoneal wall in
vivo.

The proliferation capacity in
miscellaneous tumors, including those of the

pancreas, bladder, kidney, brain, and parotid
gland, and a Ewing sarcoma exhibited the intricate
gland formations containing proliferating cells in
many of these cultured tumors.

5   It is important to note that distinctions
can be observed in the prepared autoradiograms
between proliferating malignant cells and normal
cell types, such as for the breast tumor epithelial
cells and normal stromal cells.

10   An additional important observation in
these studies is that normal tissues culture and
proliferate well. Explanted tumor and adjacent
normal tissue from the breast of patient 431 were
compared. Extensive cell proliferations were noted

15   to be present in the normal tissues. However, a
higher level of tissue organization was observed to
be maintained in the normal tissues. With this
system it is now possible to compare tumor and
normal biology-for example, nutritional

20   requirements, growth factor requirements, and
metabolic pathways. Also of critical importance,
it is now possible to compare the antitumor
selectivity of potential neoplastic agents by
comparing tumor and normal response to drugs, using

25   cell proliferation as an end point as described in
Example 3.

               These results have demonstrated a
generalized system for measurement of proliferation
capacity for all the major types of human tissues

30   in relatively long-term culture. As mentioned
above, all cultures described in this report have
been vitro for 14 days, which is a relatively long
period. Greater periods of culture can be achieved
with most tissue specimens (data not shown).

35   Greater than 90% of surgical specimens can be
cultured and analyzed for proliferative capacity

with this system.

This native-state culture system, with
the aid of polarization microscopy, allows a high
probability of detecting potential proliferative
cells.

For image analysis of proliferating
cells, a video camera was attached to the
microscope. Autoradiograms prepared as above using
breast carcinoma tissue were then viewed under
polarizing light without bright-field light,
thereby visualizing only the radioactive cells
which have exposed silver grains of the nuclear-
track emulsion. The radioactive cells brightly
reflect the polarized light. The resulting image
was analyzed by a computer-assisted image analysis
apparatus in which the image was first digitized by
a digitizer board, and then the area of brightness
corresponding to the number of labeled or bright
cells was calculated as the area of enhanced pixels
by the Fas-Com version of the P-See program (The
Microworks, Del Mar, CA) run on an IBM PC XT
compatible computer. The area of enhanced pixels
is proportional to the number of labeled cells.

With the image analysis system the
autoradiograms were automatically analyzed for the
number of labeled proliferating cells. With the
bright-field and polarized light microscopy, the
labeled cells of a cultured breast tumor appear
bright green. With epi-illumination polarization
microscopy using polarized light without bright-
field, only the labeled cells were visualized. The
image of the labeled cells was then digitized
through a video camera and the P-See program. The
area of brightness or enhanced pixels was then
automatically determined by the Fas-Com program.
The area of enhanced pixels is proportional to the

number of labeled cells, enabling the automatic
counting of labeled, proliferating cells.

5
An important aspect of the culture system
is the use of a flexible extracellular-matrix-
containing gel on which to ex-plant the tumors.
Other investigators have noted that flexible
extracellular-matrix-containing substrata are
critical to growth and function of differentiated
cells. Li et al., Proc. Natl. Acad. Sci. USA,

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84:136-40 (1987); Davis et al., Science, 236:1106-9
(197); Schaefer et al., Cancer Res., 43:279-86
(1983); Schaefer et al., Differentiation, 25:185-92
(1983); and Leighton, J., in Tissue Culture Methods
and Applications, eds., Kruse et al., (Academic,

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New York), pp. 367-71 (1973).

The general principles here are
applicable to all types of human tissues, allowing
the accumulation of potential important biological
and clinically prognostic information. In

20
addition, it should be noted that many of these
tumors have high capabilities of cell
proliferation. The eventual understanding of the
deregulation permissive for such proliferation
should be facilitated with the system described

25
here and allow us a deeper understanding of the
changes occurring in oncogenesis.

2.   Determining Cell Viability and
      Proliferative Capacity in Native-
      State Tissue Culture

30
Tumor tissue specimens from a patient
having breast carcinoma were obtained as described
in Example 1, divided into 1 mm diameter pieces,
and were each placed onto a flexible gel matrix to
form a three dimensional culture. Duplicate

35
cultures are prepared of each specimen, and 8
microcuries (uCi) of either 35S-methionine or ^{3}H-

thymidine was added to each culture that includes 2 milliliters (ml) of culture medium containing the added radiolabel. The labeled cultures were maintained as before for four days, the excess

5 radiolabel was then rinsed off of each cultured specimen using a series of phosphate-buffered saline (PBS) rinses and the cultures were each processed for histological and autoradiographic visualization as described in Example 1.

10 The prepared cultures were then analyzed by using the computer program Fas-Com for analysis after digitizing as described in Example 1. The measure of 35S-methionine incorporated into cultured cells allows the determination of cellular

15 protein synthesis and is therefore a measure of cell viability. The measure of ^{3}H-thymidine incorporation into cultured cells allows the determination of DNA synthesis and is therefore a measure of cell proliferation.

20 Tissue specimens cultured in the presence of 35S-methionine or ^{3}H-thymidine incorporated radiolabel in the portions of the tissue containing viable or proliferating cells, respectively, or both. Non-viable or non-proliferating cells did

25 not incorporate their respective labels and did not present silver grains on visual inspection of prepared specimens, nor present proliferating cells as bright green objects when analyzed in the epi-illumination polarization system.

30 The extent of proliferation measured by the in vitro native-state culturing system correlates with the grade and stage of the tumor: the more malignant the tumor, the higher the proliferation measured in vitro. The extent of

35 proliferation can be expressed as a growth fraction index (GFI), measured as the percentage of

proliferating cells present in a population of the
total number of cells present in a selected field
viewed by the microscope. Therefore measured GFI
can be used to predict the clinical progression of
the human cancer tissue tested. The Fas-Com
program analysis provides a quantitative means that
is semi-automated to determine the proliferative
capacity or viability of a tumor, and is ideally
suited to provide GFI data.

Proliferation analyses were conducted on
numerous explanted breast tumor tissues graded as
metastatic or primary, and also graded as poorly or
moderately differentiated to generate an average
GFI for each type of tumor tested. Whereas
metastatic tumors averaged 0.437 ± 0.149 GFI,
primary tumors exhibited a lower average of 0.282 ±
0.138 GFI. Similarly, whereas poorly
differentiated tumors averaged 0.372 ± 0.150 GFI,
moderately differentiated tumors averaged 0.220 ±
0.094 GFI. The results indicate that GFI
correlates with tumor severity and clinical
prognosis.

3. Determining Drug Response Using The
Native-State System For Measuring
Viability And Proliferative Capacity
Of Tissues In Vitro

Tissue specimen cultures were established
using various tumor tissues as described in Example
2. After the fourth day of culturing, multiple
cultures of each tissue were further cultured in
the presence of a drug indicated below at the
indicated concentrations for an exposure time of 24
hours.

Cultures were then washed in culture
medium to remove excess drug, cultured in the
absence of drug for 3 days to allow the cells to

recover from transient drug effects, and were then labeled as described in Example 2. After labeling, the cultures were processed as before in Example 1 to visualize the degree of viability and

5   proliferative capacity in the specimens when cultured in the presence of the drug.

The cells cultured from the ovarian carcinoma tissue of a patient having ovarian carcinoma were tested by the above methods for cell

10   proliferation in the presence of cisplatin at 1.5 μg/ml, 5-fluorouracil at 4 μg/ml, melphalan at 10 μg/ml, methotrexate at 22.5 μg/ml or thiotepa at 60 μg/ml. The results showed a decrease in the detectable signal generated by both ^{3}H-thymidine and

15   35S-methionine incorporation, indicating an inhibition of both proliferation and viability. A diminution in cell proliferation is expressed as a decrease in the GFI, when compared in the GFI for the same specimen cultured in the absence of the

20   drug. A diminution of cell proliferation was observed in an amount of 90% using cisplatin, 99% using 5-fluorouracil, 70% using melphalan, 70% using methotrexate and 90% using thiotepa. Patient S.D. having ovarian carcinoma produced explanted

25   tumor tissue that was inhibited by more than 70% in cell proliferation by melphalan at 10 μg/ml, responded to therapy using melphalan, and exhibited a decreased in tumor size during treatments. Therefore a clinical correlation was demonstrated

30   between _in vivo_ responsiveness of the tumor to the drug and the _in vitro_ native-state drug responsiveness.

The cells cultured from the tissue of a patient having breast carcinoma were tested as

35   above in the presence of drug, and resulted in the following diminutions in cell proliferation shown

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in the parenthesis: Adriamycin at 290 ng/ml (90%),
5-fluorouracil at 4 μg/ml (90%), melphalan at 1
μg/ml (80%), methotrexate at 2.25 μg/ml (70%) or
vincristine at 23 μg/ml (70%).

5   Patient D.H. was diagnosed as having
breast carcinoma and was determined to be non-
responsive to _in vivo_ therapy with either 5-
fluorouracil or adriamycin. Cellular proliferation
of patient D.H.'s breast carcinoma tissue was not
10   inhibited significantly (i.e. greater than 90%
diminution) by culturing as above in the presence
of either 5-fluorouracil or adriamycin. Therefore,
there was a clinical correlation between _in vivo_
responsiveness and _in vitro_ diminutions of cell
15   proliferation.

The cells cultured from the cancerous
tissue from a patient having colon/rectal cancer
were tested as above in the presence of the various
indicated drugs, and resulted in the following
20   diminutions in cell proliferation shown in the
parenthesis: 5-fluorouracil at 4 μg/ml (90%),
mitomycin C at 1 μg/ml (90%), and BCNU at 2 μg/ml
(90%). Patient V.S. was diagnosed as having colon
carcinoma and was determined to be non-responsive
25   to _in vivo_ therapy using 5-fluorouracil. Cellular
proliferation of V.S.'s colon carcinoma cells was
not inhibited significantly, i.e., greater than
90%, by culturing the explants as above in the
presence of 5-fluorouracil. A clinical correlation
30   was again observed between _in vivo_ and _in vitro_
responsiveness.

The above results show that cellular
proliferation can be used as a measure of a tumor's
drug responsiveness, where an active drug inhibits
35   cellular proliferation. Where cells are not in a
proliferative state, the viability of the tumor

tissue as measured by 35S-methionine incorporation can be used to indicate the tumor's drug responsiveness. The extent of viability can also be used to determine an endpoint for maximum

5 responsiveness.

The foregoing description and the examples are intended as illustrative and are not to be taken as limiting. Still other variations within the spirit and scope of this invention are

10 possible and will readily present themselves to those skilled in the art.

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What Is Claimed Is:

     1. A method for measuring the tumor specific effects of an agent on proliferation and viability, which method comprises:

     a) histoculturing, in separate containers, first and second portions of tumor tissue and analogous normal tissue samples;

     b) exposing said samples to said agent;

     c) treating said first portions of said samples with DNA-synthesis marker and treating said second portions of said samples with a protein-synthesis marker;

     d) culturing said treated portions of said samples for a predetermined time period; and

     e) measuring the amount of DNA-synthesis marker and protein-synthesis marker incorporation in said first and second portions, respectively, and thereby the tumor specific effects of said compound on cell proliferation and viability.

     2. The method of claim 1 wherein the DNA-synthesis-marker is ^{3}H-thymidine and the protein synthesis-marker is ^{35}S-thymidine.

     3. The method of claim 1 wherein said predetermined time period of step (d) is two to about 5 days.

     4. The method of claim 1 wherein said first and second portions of said samples are cultured 2 to about 6 days prior to said exposing of step (b).

     5. A method for measuring the aggressiveness of a tumor, which method comprises:

     a) histoculturing a sample of tumor cells;

b)   treating the sample with a DNA-synthesis marker;

c)   culturing said treated sample for a predetermined time period; and

d)   determining the percent of tumor cells having incorporated therein said DNA-synthesis marker, and thereby the aggressiveness of said tumor.

6.   The method of claim 5 wherein said DNA-synthesis marker is ^{3}H-deoxythimidine.

7.   A kit for the in vitro determination of the viability and proliferation of cells, which kit comprises a protein synthesis marker and a DNA synthesis marker.

8.   The kit of claim 7 wherein said protein synthesis marker is ^{35}S-methionine and said DNA synthesis marker is ^{3}H-deoxythymidine.\

9.   The kit of claim 7 further containing an agent cytotoxic for tumor.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/03719

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12Q 1/68
U.S. Cl.: 435/6

II. FIELDS SEARCHED

Minimum Documentation Searched [7]	
Classification System	Classification Symbols
U.S.	435/6,68,91,240.23,810 436/63,64,86,106,813,815 935/11,16,36,77,86

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Cancer Research, Volume 42, published in 1982, F. R. Althaus et al., "Chemical Quantitation of Unscheduled DNA Synthesis in Cultured Hepatocytes as an Assay for the Rapid Screening of Potential Chemical Carcinogens," pages 3010-3015, see especially the Introduction section on page 3010.	1-8
X	Journal of Toxicology and Environmental Health, Volume 16, published in 1985, C.H. van Aswegen et al., "Influence of Phomopsin and Ivalin on Steroid Hormone Binding and Growth of MCF-7 Human Breast Cancer Cells," pages 13-23, see especially the Abstract on page 13.	1-9
X,P	Proceedings of the National Academy of Sciences (USA), Volume 86, published in 1989, March, R.M. Hoffman, et al., "A General Native-state method for determination of proliferation capacity of human normal and tumor tissues in vitro," pages 2013-2017, see especially the Abstract on page 2013.	1-9

* Special categories of cited documents: [10]
"A" document defining the general state of the art which is not considered to be of particular relevance
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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
09 FEBRUARY 1990 (09.02.90)	23 FEB 1990
International Searching Authority	Signature of Authorized Officer
ISA/US	Ardin Marschel

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Folia biologica (Praha), Volume 28, published in 1982, R. Stetina, et al., "Effect of Aflatoxin B_1 on Growth of Normal and Malignant Rat Fibroblasts in Tissue Culture," pages 193-210, see especially the Results section, bridging paragraph on pages 199-200, and the section on ^{3}H-thymidine incorporation on pages 195-196, bridging paragraph.	1-9
Y	Experimental Cell Research, Volume 88, published in 1974, J.E. Trosko, et al., "A Sensitive Method to Measure Physical and Chemical Carcinogen-induced "Unscheduled DNA Synthesis" in Rapidly Dividing Eukaryotic Cells," pages 47-55, see especially the Materials and Methods section (Tissue Culture and Experimental culture procedures) on page 48.	1-9
Y	The Journal of Histotechnology, Volume 9, Number 1, published in 1986, A. Richters, "Advantages of Combining Histopathology, Tissue Culture, and Ultrastructure in Studying Disease Processes," pages 21-22, see especially the Introduction on page 21.	1-9
A	US, A, 4,711,856 (SPELSBERG) 08 DECEMBER 1987 (08.12.87), see especially Figure 1.	1-9

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